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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US97/12656 <b>(22) International Filing Date:</b> 17 July 1997 (17.07.97)  <b>(30) Priority Data:</b> 08/683,242 18 July 1996 (18.07.96) US  <b>(71) Applicant:</b> THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 N. Torrey Pines Road, La Jolla, CA 92037 (US).  <b>(72) Inventors:</b> DOERNER, Peter, W.; 1410 Hornblend, San Diego, CA 92109 (US). LAMB, Christopher, J.; 6444 Farley Drive, San Diego, CA 92122 (US).  <b>(74) Agent:</b> HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF INCREASING GROWTH AND YIELD IN PLANTS  <b>(57) Abstract</b>  The invention provides a method of producing a genetically modified plant characterized as having increased growth and yield as compared to a corresponding wild-type plant comprising increasing the level of cyclin expression in the plant. Genetically modified plants characterized as having increased growth and yield are also provided.		

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**METHOD OF INCREASING GROWTH AND YIELD IN PLANTS****5 Field of the Invention**

The present invention relates generally to plant genetic engineering, and specifically to a method for producing genetically engineered plants characterized as having increased growth and yield.

**10 Background of the Invention**

For each plant species, there exists a wide discrepancy in plant growth due to environmental conditions. Under most conditions, the maximum growth potential of a plant is not realized. Plant breeding has demonstrated that a plant's resources can be redirected to individual organs to enhance growth.

- 15 Genetic engineering of plants, which entails the isolation and manipulation of genetic material, e.g., DNA or RNA, and the subsequent introduction of that material into a plant or plant cells, has changed plant breeding and agriculture considerably over recent years. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals,
- 20 chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques.

The ability to manipulate gene expression provides a means of producing new characteristics in transformed plants. For example, the ability to increase the size of a plant's root system would permit increased nutrient assimilation from the soil.

- 25 Moreover, the ability to increase leaf growth would increase the capacity of a plant to assimilate solar energy. Obviously, the ability to control the growth of an entire plant, or specific target organs thereof would be very desirable.

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Summary of the Invention

The present invention is based on the discovery that increased growth and yield in plants can be achieved by elevating the level of cyclin expression.

In a first embodiment, the invention provides a method of producing a genetically modified plant characterized as having increased growth and yield as compared to a corresponding wild-type plant. The method comprises contacting plant cells with nucleic acid encoding a cyclin protein, wherein the nucleic acid is operably associated with a regulatory sequence, to obtain transformed plant cells; producing plants from the transformed plant cells; and selecting a plant exhibiting said increased yield. The cyclin-  
10 encoding nucleic acid preferably encodes the cyclin cyclinAAt.

In another embodiment, the invention provides a method of producing a plant characterized as having increased yield, the method comprising contacting a plant with an agent which elevates cyclin expression above cyclin expression in a plant not contacted with the agent. The agent may be a transcription factor or a chemical agent  
15 which induces an endogenous cyclin promoter or other chemically inducible promoter driving expression as the cyclin transgene.

The invention also provides plants, plant tissue and seeds produced by the methods of the invention.

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Brief Description of the Drawing

Figure 1 shows steady state levels of *cdc2aAt* mRNA and p34 protein, panel a; *cyclaAt* mRNA during IAA induction of lateral root meristems, panel b; *cyclaAt* mRNA in selected non-induced transgenic lines, panel c; normalized transcript levels relative to wild-type are indicated. Col-0, wild-type; 1A2, 2A5, 4A3, 11A1: T2 homozygous; 6A, 7A, 8A: T1 heterozygous transgenic lines.

Figure 2 shows an *in situ* hybridization analysis of *cdc2aAt* and *cyclaAt* transcripts in root apices and developing lateral roots. Panels a-d show cross sections of quiescent roots (panels a,b) or proliferating cells in primordia (panels c,d) that were hybridized to *cdc2aAt* (a) or *cyclaAt* (b-d) anti-sense probes. Panels e, f show *cyclaAt* mRNA abundance in contiguous meristematic cell files in root apices. Transcript accumulation is indicated by silver grain deposition and visualized by indirect red illumination. Scale bar is 10  $\mu$ m in a-d, 5  $\mu$ m in e, f. fc, founder cell accumulating *cyclaAt* transcripts; p, pericycle cell layer; r, towards the root apex; s, towards the shoot.

Figure 3 shows increased root growth rate in *Arabidopsis thaliana* (*A.thaliana*) ectopically expressing *cyclaAt* cyclin. Panel a. Wild-type (left) or transgenic line 6A (T1 generation) containing the *cdc2aAt::cyclaAt* gene fusion (right). Arabidopsis seed were plated on MS (3% sucrose) agar and grown in a vertical orientation for 7 d. Plants transformed with the vector alone or with unrelated promoter::uidA constructs or with a *cdc2aAt::cyclaAt* fusion in which the *cdc2aAt* 5' untranslated leader was interrupted by a DS transposon insertion did not show this phenotype. Panel b. wild-type (left) or transgenic line 6A (T1 generation) (right) 6 d after IAA induction of lateral roots. One week-old seedlings grown hydroponically were treated with 10  $\mu$ M IAA<sub>eff</sub> to stimulate lateral root development.

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Description of the Preferred Embodiments

The present invention provides methods for increasing the yield of a plant, such as a agricultural crop, by elevating the cyclin expression level in the plant. Increased cyclin expression in plant cells competent to divide results in increased plant growth.

- 5 In a preferred embodiment, the invention provides a method for producing a genetically modified plant characterized as having increased yield as compared to a plant which has not been genetically modified (e.g., a wild-type plant). The method comprises contacting plant cells with nucleic acid encoding a cyclin protein, wherein the nucleic acid is operably associated with a regulatory sequence to obtain transformed plant cells;
- 10 producing plants from the transformed plant cells; and thereafter selecting a plant exhibiting increased growth and yield.

As used herein, the term "yield" or "plant yield" refers to increased crop growth, and/or increased biomass. In one embodiment, increased yield results from increased growth rate and increased root size. In another embodiment, increased yield is derived from

15 shoot growth. In still another embodiment, increased yield is derived from fruit growth.

The term "genetic modification" as used herein refers to the introduction of one or more exogenous nucleic acid sequences, e.g., cyclin encoding sequences, as well as regulatory sequences, into one or more plant cells, which can generate whole, sexually competent, viable plants. The term "genetically modified" as used herein refers to a

20 plant which has been generated through the aforementioned process. Genetically modified plants of the invention are capable of self-pollinating or cross-pollinating with other plants of the same species so that the foreign gene, carried in the germ line, can be inserted into or bred into agriculturally useful plant varieties. The term "plant cell" as used herein refers to protoplasts, gamete producing cells, and cells which regenerate

25 into whole plants.

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As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells, such as plant tissue or plant seed. Plantlets are also included within the meaning of "plant". Plants included in the invention are any plants amenable to transformation techniques, including gymnosperms and angiosperms, both  
5 monocotyledons and dicotyledons.

Examples of monocotyledonous angiosperms include, but are not limited to, asparagus, field and sweet corn, barley, wheat, rice, sorghum, onion, pearl millet, rye and oats and other cereal grains. Examples of dicotyledonous angiosperms include, but are not limited to tomato, tobacco, cotton, rapeseed, field beans, soybeans, peppers, lettuce, peas, alfalfa,  
10 clover, cole crops or *Brassica oleracea* (e.g., cabbage, broccoli, cauliflower, brussel sprouts), radish, carrot, beets, eggplant, spinach, cucumber, squash, melons, cantaloupe, sunflowers and various ornamentals. Examples of woody species include poplar, pine, sequoia, cedar, oak, etc.

The term "exogeneous nucleic acid sequence" as used herein refers to a nucleic acid  
15 foreign to the recipient plant host or, native to the host if the native nucleic acid is substantially modified from its original form. For example, the term includes a nucleic acid originating in the host species, where such sequence is operably linked to a promoter that differs from the natural or wild-type promoter. In the broad method of the invention, at least one nucleic acid sequence encoding cyclin is operably linked with a promoter.  
20 It may be desirable to introduce more than one copy of cyclin polynucleotide into a plant for enhanced cyclin expression. For example, multiple copies of a cyclin polynucleotide would have the effect of increasing production of cyclin even further in the plant.

The term "regulatory sequence" as used herein refers to a nucleic acid sequence capable of controlling the transcription of an operably associated gene. Therefore, placing a gene  
25 under the regulatory control of a promoter or a regulatory element means positioning the gene such that the expression of the gene is controlled by the regulatory sequence(s). In general, promoters are found positioned 5' (upstream) of the genes that they control.



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Thus, in the construction of promoter gene combinations, the promoter is preferably positioned upstream of the gene and at a distance from the transcription start site that approximates the distance between the promoter and the gene it controls in the natural setting. As is known in the art, some variation in this distance can be tolerated without  
5 loss of promoter function. Similarly, the preferred positioning of a regulatory element, such as an enhancer, with respect to a heterologous gene placed under its control reflects its natural position relative to the structural gene it naturally regulates.

Cyclin-encoding nucleic acids utilized in the present invention include nucleic acids encoding mitotic cyclins such as, for example, cyclin B; nucleic acids encoding S- phase  
10 cyclins such as, for example cyclin A, and nucleic acids encoding G1 phase cyclins. Specific cyclins which can be utilized herein include cyc1aAt, cyc3aAt, cyc3bAt, cycd1, cycd2 and the like. Preferably, the nucleic acid used in the method of the invention encodes the cyc1aAt protein (Genebank Accession No. X62279).

Genetically modified plants of the present invention are produced by contacting a plant  
15 cell with a nucleic acid sequence encoding the desired cyclin. To be effective once introduced into plant cells, the cyclin-encoding nucleic acid must be operably associated with a promoter which is effective in plant cells to cause transcription of the cyclin transgene. Additionally, a polyadenylation sequence or transcription control sequence, also recognized in plant cells, may also be employed. It is preferred that the nucleic acid  
20 be introduced via a vector and that the vector harboring the nucleic acid sequence also contain one or more selectable marker genes so that the transformed cells can be selected from non-transformed cells in culture, as described herein.

The term "operably associated" refers to functional linkage between a regulatory sequence, preferably a promoter sequence, and the cyclin-encoding nucleic acid sequence  
25 regulated by the promoter. The operably linked promoter controls the expression of the cyclin nucleic acid sequence.

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The expression of cyclin genes employed in the present invention may be driven by a number of promoters. Although the endogenous, or native promoter of a structural gene of interest may be utilized for transcriptional regulation of the gene, preferably, the promoter is a foreign regulatory sequence.

- 5 When it is desired to increase growth and yield in the whole plant, cyclin expression should be directed to all cells in the plant which are capable of dividing. This can be accomplished by using a promoter active in all meristems. Such promoters include, for example, the *cdc2a* promoter and the *cyc07* promoter. (See for example, Ito *et al.*, *Plant Mol. Biol.*, 24:863, 1994; Martinez *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:7360, 1992;
- 10 Medford *et al.*, *Plant Cell*, 3:359, 1991; Terada *et al.*, *Plant Journal*, 3:241, 1993; Wissenbach *et al.*, *Plant Journal*, 4:411, 1993).

- When it is desired to increase growth and yield in a specific organ, cyclin expression should be targeted to the appropriate meristem, e.g., the shoot meristem, the floral meristem, the root meristem etc. This can be accomplished by using a tissue specific
- 15 promoter. Examples of tissue specific promoters active in shoot meristems are described in Atanassova *et al.*, *Plant Journal*, 2:291, 1992 and Medford *et al.*, *Plant Cell*, 3:359, 1991. Examples of tissue specific promoters active in floral meristems are the promoters of the *agamous* and *apetala 1* genes are described in Bowman *et al.*, *Plant Cell*, 3:749, 1991; and Mandel *et al.*, *Nature*, 360:273, 1992.

- 20 The particular promoter selected should be capable of causing sufficient cyclin expression to cause increased yield and/or increased biomass. It should be understood that cyclin expression can be altered in cells that are competent to divide. The promoters used in the vector constructs of the present invention may be modified, if desired, to affect their control characteristics.
- 25 Optionally, a selectable marker may be associated with the cyclin-encoding nucleic acid. As used herein, the term "marker" refers to a gene encoding a trait or a phenotype which

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permits the selection of, or the screening for, a plant or plant cell containing the marker. Preferably, the marker gene is an antibiotic resistance gene whereby the appropriate antibiotic can be used to select for transformed cells from among cells that are not transformed. Examples of suitable selectable markers include adenosine deaminase, 5 dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase, xanthine-guanine phospho-ribosyltransferase and amino-glycoside 3'-O-phosphotransferase II. Other suitable markers will be known to those of skill in the art.

To commence a transformation process in accordance with the present invention, it is first necessary to construct a suitable vector and properly introduce it into the plant cell. 10 Vector(s) employed in the present invention for transformation of a plant cell include a cyclin-encoding nucleic acid sequence operably associated with a promoter. Details of the construction of vectors utilized herein are known to those skilled in the art of plant genetic engineering.

Cyclin-encoding nucleic acid sequences utilized in the present invention can be 15 introduced into plant cells using Ti plasmids of *Agrobacterium tumefaciens* (*A. tumefaciens*), root-inducing (Ri) plasmids of *Agrobacterium rhizogenes* (*A. rhizogenes*), and plant virus vectors. (For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology*, 20 2d Ed., Blackie, London, Ch. 7-9, and Horsch *et al.*, *Science*, 227:1229, 1985, both incorporated herein by reference). In addition to plant transformation vectors derived from the Ti or Ri plasmids of *Agrobacterium*, alternative methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, transformation using viruses or pollen and the use of microprojection.

25 One of skill in the art will be able to select an appropriate vector for introducing the cyclin-encoding nucleic acid sequence in a relatively intact state. Thus, any vector which will produce a plant carrying the introduced cyclin-encoding nucleic acid should be

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sufficient. Even use of a naked piece of DNA would be expected to confer the properties of this invention, though at low efficiency. The selection of the vector, or whether to use a vector, is typically guided by the method of transformation selected.

The transformation of plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. (See, for example, *Methods of Enzymology*, Vol. 153, 1987, Wu and Grossman, Eds., Academic Press, incorporated herein by reference). As used herein, the term "transformation" means alteration of the genotype of a host plant by the introduction of cyclin-nucleic acid sequence.

- 10 For example, a cyclin-encoding nucleic acid can be introduced into a plant cell utilizing *A. tumefaciens* containing the Ti plasmid, as mentioned briefly above. In using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of *Agrobacterium* as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is also preferred that the
- 15 *Agrobacterium* harbor a binary Ti plasmid system. Such a binary system comprises 1) a first Ti plasmid having a virulence region essential for the introduction of transfer DNA (T-DNA) into plants, and 2) a chimeric plasmid. The chimeric plasmid contains at least one border region of the T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. Binary Ti plasmid systems have been shown effective in
- 20 the transformation of plant cells (De Framond, *Biotechnology*, 1:262, 1983; Hoekema *et al.*, *Nature*, 303:179, 1983). Such a binary system is preferred because it does not require integration into the Ti plasmid of *A. tumefaciens*, which is an older methodology.

Methods involving the use of *Agrobacterium* in transformation according to the present invention include, but are not limited to: 1) co-cultivation of *Agrobacterium* with

25 cultured isolated protoplasts; 2) transformation of plant cells or tissues with *Agrobacterium*; or 3) transformation of seeds, apices or meristems with *Agrobacterium*.

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In addition, gene transfer can be accomplished by *in planta* transformation by *Agrobacterium*, as described by Bechtold *et al.*, (*C.R. Acad. Sci. Paris*, 316:1194, 1993) and exemplified in the Examples herein. This approach is based on the vacuum infiltration of a suspension of *Agrobacterium* cells.

- 5 The preferred method of introducing a cyclin-encoding nucleic acid into plant cells is to infect such plant cells, an explant, a meristem or a seed, with transformed *A. tumefaciens* as described above. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants.

- Alternatively, cyclin-encoding nucleic acid can be introduced into a plant cell using  
10 mechanical or chemical means. For example, the nucleic acid can be mechanically transferred into the plant cell by microinjection using a micropipette. Alternatively, the nucleic acid may be transferred into the plant cell by using polyethylene glycol which forms a precipitation complex with genetic material that is taken up by the cell.

- 15 Cyclin-encoding nucleic acid can also be introduced into plant cells by electroporation (Fromm *et al.*, *Proc. Natl. Acad. Sci., U.S.A.*, 82:5824, 1985, which is incorporated herein by reference). In this technique, plant protoplasts are electroporated in the presence of vectors or nucleic acids containing the relevant nucleic acid sequences. Electrical impulses of high field strength reversibly permeabilize membranes allowing  
20 the introduction of nucleic acids. Electroporated plant protoplasts reform the cell wall, divide and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers as described herein.

- Another method for introducing a cyclin-encoding nucleic acid into a plant cell is high velocity ballistic penetration by small particles with the nucleic acid to be introduced  
25 contained either within the matrix of such particles, or on the surface thereof (Klein *et al.*, *Nature* 327:70, 1987). Bombardment transformation methods are also described in Sanford *et al.* (*Techniques* 3:3-16, 1991) and Klein *et al.* (*Bio/Techniques* 10:286, 1992).

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Although, typically only a single introduction of a new nucleic acid sequence is required, this method particularly provides for multiple introductions.

- Cauliflower mosaic virus (CaMV) may also be used as a vector for introducing nucleic acid into plant cells (US Patent No. 4,407,956). CaMV viral DNA genome is inserted
- 5 into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid again may be cloned and further modified by introduction of the desired nucleic acid sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.
- 10 As used herein, the term "contacting" refers to any means of introducing a cyclin-encoding nucleic acid into a plant cell, including chemical and physical means as described above. Preferably, contacting refers to introducing the nucleic acid or vector containing the nucleic acid into plant cells (including an explant, a meristem or a seed), via *A. tumefaciens* transformed with the cyclin-encoding nucleic acid as described above.
- 15 Normally, a plant cell is regenerated to obtain a whole plant from the transformation process. The immediate product of the transformation is referred to as a "transgenote". The term "growing" or "regeneration" as used herein means growing a whole plant from a plant cell, a group of plant cells, a plant part (including seeds), or a plant piece (e.g., from a protoplast, callus, or tissue part).
- 20 Regeneration from protoplasts varies from species to species of plants, but generally a suspension of protoplasts is first made. In certain species, embryo formation can then be induced from the protoplast suspension. The culture media will generally contain various amino acids and hormones, necessary for growth and regeneration. Examples of hormones utilized include auxins and cytokinins. Efficient regeneration will depend
- 25 on the medium, on the genotype, and on the history of the culture. If these variables are controlled, regeneration is reproducible.

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Regeneration also occurs from plant callus, explants, organs or parts. Transformation can be performed in the context of organ or plant part regeneration. (see *Methods in Enzymology*, Vol. 118 and Klee *et al.*, *Annual Review of Plant Physiology*, 38:467, 1987). Utilizing the leaf disk-transformation-regeneration method of Horsch *et al.*,  
5 *Science*, 227:1229, 1985, disks are cultured on selective media, followed by shoot formation in about 2-4 weeks. Shoots that develop are excised from calli and transplanted to appropriate root-inducing selective medium. Rooted plantlets are transplanted to soil as soon as possible after roots appear. The plantlets can be repotted as required, until reaching maturity.

- 10 In vegetatively propagated crops, the mature transgenic plants are propagated by utilizing cuttings or tissue culture techniques to produce multiple identical plants. Selection of desirable transgenotes is made and new varieties are obtained and propagated vegetatively for commercial use.

- In seed propagated crops, mature transgenic plants can be self crossed to produce a  
15 homozygous inbred plant. The resulting inbred plant produces seed containing the newly introduced foreign gene(s). These seeds can be grown to produce plants that would produce the selected phenotype. *e.g.* increased yield.

- Parts obtained from regenerated plant, such as flowers, seeds, leaves, branches, roots, fruit, and the like are included in the invention, provided that these parts comprise plant  
20 cells that have been transformed as described. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

- Plants exhibiting increased growth and/or yield as compared with wild-type plants can be selected by visual observation. The invention includes plants produced by the  
25 method of the invention, as well as plant tissue and seeds.

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In another embodiment, the invention provides a method of producing a plant characterized as having increased growth and yield by contacting a plant capable of increased yield with a cyclin promoter-inducing amount of an agent which induces cyclin gene expression. Induction of cyclin gene expression results in production of a plant  
5 having increased yield as compared to a plant not contacted with the agent.

A "plant capable of increased yield" refers to a plant that can be induced to express its endogenous cyclin gene to achieve increased yield. The term "promoter inducing amount" refers to that amount of an agent necessary to elevate cyclin gene expression above cyclin expression in a plant cell not contacted with the agent, by stimulating the  
10 endogenous cyclin promoter. For example, a transcription factor or a chemical agent may be used to elevate gene expression from native cyclin promoter, thus inducing the promoter and cyclin gene expression.

The invention also provides a method of providing increased transcription of a nucleic acid sequence in a selected tissue. The method comprises growing a plant having  
15 integrated in its genome a nucleic acid construct comprising, an exogenous gene encoding a cyclin protein, said gene operably associated with a tissue specific whereby transcription of said gene is increased in said selected tissue.

Plant development is plastic with post-embryonic organogenesis mediated by meristems (Steeves and Sussex, *Patterns in Plant Development*, 1-388 (Press Syndicate of the  
20 University of Cambridge, Cambridge, 1989)). Although cell division is intrinsic to meristem initiation, maintenance and proliferative growth, the role of the cell cycle in regulating growth and development is unclear. To address this question, the expression of *cdc2* and cyclin genes, which encode the catalytic and regulatory subunits, respectively, of cyclin-dependent protein kinases controlling cell cycle progression  
25 (Murray and Hunt, *The Cell Cycle* (New York), 1993) were examined. Unlike *cdc2*, which is expressed not only in apical meristems but also in quiescent meristems. (Martinez *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:7360, 1992), transcripts of *cyc1aAt*



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accumulated specifically in active meristems and dividing cells immediately before cytokinesis. Ectopic expression of *cyc1aAt* under control of the *cdc2aAt* promoter in Arabidopsis plants markedly accelerated growth without altering the pattern of development or inducing neoplasia. Thus, cyclin expression is a limiting factor for growth.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

10

#### EXAMPLE 1

A full length *cyc1aAt* cyclin cDNA was placed under control of the *Arabidopsis cdc2aAt* promoter (Hemerly *et al.*, 1992, *supra*). The chimeric gene was cloned into a T-DNA transformation vector carrying the selection marker hygromycin phospho-transferase (Hyg<sup>r</sup>) and transformed into *Arabidopsis* using the vacuum-infiltration method (Bechtold and Pelletier, *Acad. Sci. Paris, Life Sci.*, 316:1194, 1993) to introduce *Agrobacterium tumefaciens*. Several independent transgenic lines having elevated steady-state levels of *cyc1aAt* mRNA showed a dramatic increase of both main and lateral root growth rate, correlated with proportionally increased fresh weight, dry mass and DNA content, but not cell size. Enhanced growth was orderly, with no observed differences in morphology and clearly not neoplastic.

Arabidopsis seedlings (ecotype Columbia) were grown in 20 ml MS medium (Murashige and Skoog, *Physiol. Plant.*, 15:473, 1962). Eight- to 10-day-old plants were transferred to MS medium buffered with 50 mM potassium phosphate, pH 5.5, and initiation of lateral roots was stimulated by addition of IAA to 10  $\mu\text{M}_{\text{eff}}$  (non-dissociated IAA). Roots were collected at the time points indicated and total RNA and protein isolated. 500 ng poly(A)<sup>+</sup> RNA was separated on 1% formaldehyde gels (Ausubel *et al.*, *Current Protocols in Molecular Biology*. Green Publishing Associates and Wiley-Interscience,

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New York, 1987), transferred to Nytran membranes (Schleicher and Schüll) and hybridized to <sup>32</sup>P-labeled probes corresponding to nucleotides (nt) 674-1004 of *cyclAAt* (Hemerly *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:3295, 1992), or nt 661-1386 of Arabidopsis *cdc2aAt* (Hirayama *et al.*, *Gene*, 105:159, 1991), followed by hybridization  
5 with nt 2576-2824 of Arabidopsis *UBQ3* (Norris *et al.*, *Plant Mol. Biol.*, 21:895, 1993) for normalization. Blots were quantified with a Molecular Dynamics Phosphorimager. *cyclAAt* is a single copy gene in Arabidopsis. Total protein was separated on 12% SDS-PAGE and transferred to PVDF membranes. p34<sup>cdc2aAt</sup> was detected with serum raised in rabbits against the peptide YFKDLGGMP (SEQ ID NO:1), corresponding to amino acids  
10 286-294, and visualized by Enhanced Chemiluminescence Assay (Amersham).

Figure 1 shows steady state levels of *cdc2aAt* mRNA and p34 protein, panel *a*; *cyclAAt* mRNA during IAA induction of lateral root meristems, panel *b*; *cyclAAt* mRNA in selected non-induced transgenic lines, panel *c*; normalized transcript levels relative to wild-type are indicated. Col-0, wild-type; 1A2, 2A5, 4A3, 11A1: T2 homozygous; 6A,  
15 7A, 8A: T1 heterozygous transgenic lines. *cyclAAt* mRNA levels in the lines 4A3, 6A, 7A, 8A, and 3A exceeded those of IAA induced wild-type roots.

The levels of *cdc2* mRNA and p34<sup>cdc2</sup> protein per cell did not markedly change following stimulation of lateral root initiation by the auxin indoleacetic acid (IAA) (Fig. 1, panel *a*). Hence, while *cdc2* expression is correlated with the competence to divide, root growth  
20 and initiation of lateral roots do not appear to be limited by the abundance of the cyclin-dependent protein kinase p34 catalytic subunit and, moreover, ectopic expression of *cdc2* in transgenic Arabidopsis failed to perturb growth or development (Hemerly *et al.*, *EMBO J.*, 14:3925, 1995).

In contrast, IAA treatment of Arabidopsis roots induced the expression of several *cyc*  
25 genes from low basal levels and in particular *cyclAAt* mRNA, which encodes a mitotic cyclin (Hemerly *et al.*, *supra*), exhibited a rapid 15 to 20-fold increase (Fig. 1, panel *b*).

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Figure 2 shows an *in situ* hybridization analysis of *cdc2aAt* and *cyclaAt* transcripts in root apices and developing lateral roots. Panels *a-d* show cross sections of quiescent roots (panels *a,b*) or proliferating cells in primordia (panels *c,d*) that were hybridized to *cdc2aAt* (*a*) or *cyclaAt* (*b-d*) anti-sense probes. Panels *e,f* show *cyclaAt* mRNA abundance in contiguous meristematic cell files in root apices. Transcript accumulation is indicated by silver grain deposition and visualized by indirect red illumination. Scale bar is 10  $\mu$ m in *a-d*, 5  $\mu$ m in *e*. fc, founder cell accumulating *cyclaAt* transcripts; p, pericycle cell layer; r, towards the root apex; s, towards the shoot.

Tissue samples were processed for *in situ* hybridization to examine expression of cyclin transcripts. The samples were treated with 10  $\mu$ M IAA. After 8 or 24 h incubation, radish (*Raphanus sativa* var Scarlet Globe) roots were processed as described (Drews *et al.*, *Cell*, 65:991, 1991). Sections (8  $\mu$ m) were hybridized to a  $^{33}$ P-labeled RNA probe, corresponding to nt 674-1004 of *cyclaAt* (Hemerly *et al.*, *supra*) (Fig. 2, panels *b-e*) or to a  $^{35}$ S-labeled probe used in a corresponding to nt 661-1386 of *cdc2aAt* (Hirayama *et al.*, *supra*), for 14 h at 48 °C in 50% formamide. After hybridization, the final washes were for 1 h at 58°C in 0.015 m NaCl and slides were then exposed for 3 weeks (*cyclaAt*) or 5d (*cdc2aAt*). After developing, silver grains were illuminated laterally with red light, specimens were visualized by phase contrast and double exposures were taken on FUJI Velvia film. Images were assembled in ADOBE Photoshop. For the analysis summarized in Fig. 2, panel *f*, silver grains were counted and cell size measured in the cell file shown in Fig. 2, panel *e*.

*In situ* hybridization showed that, unlike *cdc2*, *cyclaAt* transcripts were not detected in quiescent pericycle cells, but accumulated in single, cytoplasmically dense cells of incipient lateral root primordia, and in the emergent organ *cyclaAt* was expressed exclusively in the meristem (Fig. 2, panels *a-d*). Moreover, crucifer roots consisted of long cell files that arise by transverse divisions followed by longitudinal expansion (Dolan *et al.*, *Development*, 119:71, 1993), and within such a contiguous spatial display of sequential cell division phases, *cyclaAt* transcripts accumulated only in large cells

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immediately prior to cytokinesis, declining to background levels in the adjacent small daughter cells (Fig. 2, panels *e, f*). A similar, stringent spatio-temporal relationship of cyclin expression and mitosis was observed in *Antirrhinum* shoot apical meristems (Fobert *et al.*, *EMBO J.*, **13**:616, 1994).

- 5 The close correlation between *cycl1aAt* expression and cell division during growth of the root apical meristem and the initiation of lateral roots, together with the pattern of *cycl1aAt* promoter activity deduced from the expression of *cycl1aAt::uidA* gene fusions in transgenic *Arabidopsis* (Ferreira *et al.*, *Plant Cell*, **6**:1763, 1994), suggested that cyclin abundance might be a key factor regulating root growth and development. To test this
- 10 hypothesis transgenic *Arabidopsis* were generated (Bechtold and Pelletier, *Acad. Sci. Paris. Life Sci.*, **316**:1194, 1993) containing *cycl1aAt* under control of the *cdc2aAt* promoter. Five transformants were obtained in which the level of *cycl1aAt* mRNA in untreated roots exceeded that observed in IAA-stimulated roots of wild-type plants (Fig. 1, panel c), and these lines were chosen for further study.
- 15 An *NheI* site was introduced in the third codon of the *cycl1aAt* cDNA by *in vitro* mutagenesis and this open reading frame subsequently ligated to the *cdc2aAt* promoter with an *in vitro* generated *XbaI* site at codon 3. This fragment was ligated into pBiB-Hyg (Becker *et al.*, *Pl. Mol. Biol.*, **20**:1195, 1992) and transfected into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, *Mol. Gen. Genet.*, **204**:383, 1986). *Arabidopsis*
- 20 *thaliana* (*A. thaliana*) (ecotype Columbia) was transformed by vacuum infiltration (Bechtold *et al.*, *supra*), and transgenic seedlings (T0 generation) were selected on MS plates containing 30 µg/ml hygromycin. 52 independent transgenic lines were obtained and elevated levels of *cycl1aAt* mRNA were detected in 9 of the 11 lines analyzed in detail. Growth assays were performed on heterozygous T1 and homozygous T2 progeny
- 25 as indicated.

Figure 3 shows increased root growth rate in *Arabidopsis* ectopically expressing *cycl1aAt* cyclin. Panel *a*. Wild-type (*left*) or transgenic line 6A (T1 generation) containing the

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*cdc2aAt::cyc1aAt* gene fusion (*right*). Arabidopsis seed were plated on MS (3% sucrose) agar and grown in a vertical orientation for 7 d. Plants transformed with the vector alone or with unrelated promoter::*uidA* constructs or with a *cdc2aAt::cyc1aAt* fusion in which the *cdc2aAt* 5' untranslated leader was interrupted by a DS transposon insertion did not  
5 show this phenotype. Panel b, wild-type (*left*) or transgenic line 6A (T1 generation) (*right*) 6 d after IAA induction of lateral roots. One week-old seedlings grown hydroponically were treated with 10  $\mu$ M IAA<sub>eff</sub> to stimulate lateral root development.

Strong expression of the *cdc2aAt::cyc1aAt* transgene caused a marked increase in the rate of organized root growth (Fig. 3, panel *a*). Homozygous or heterozygous seed were  
10 plated on MS agar and plants grown in vertical orientation for 7 days with a 16h day/ 8 h night schedule at 22°C. Four images of each plate were acquired with a Speedlight Platinum frame grabber (Lighttools Research) at 24 h intervals and root growth analyzed with NIH-Image by measuring the displacement of root apices. Following growth analysis, roots from 10 plants of each class were collected and RNA analyzed. To  
15 measure cell sizes, roots were cleared by overnight incubation in saturated chloral hydrate, visualized with Normarski optics, photographed and analyzed with NIH-Image. Statistical analysis (t-test with unpaired variances) was performed with MS Excel. Root growth in IAA-treated plants was assessed 3 and 6 d after induction by determination of fresh weight of roots excised from liquid-grown plants and then dry weight following  
20 lyophilization for 24 h. Total DNA was extracted from dried material (Ausubel *et al.*, *supra*).

In heterozygous T2 progeny, increased growth rate, measured by displacement of the apex of the main root in time-lapse photography, strictly co-segregated with transgene expression and individuals lacking the transgene grew at the same rate as wild-type  
25 plants (Table 1). The average size of epidermal, cortical, endodermal and pericycle cells was equivalent or slightly reduced in *cdc2aAt::cyc1aAt* transformants compared to wild-type plants (Table 2), and hence increased growth reflects increased cell number rather than cell size. The pattern of spontaneous lateral root initiation and overall root

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morphology were indistinguishable in wild-type and transgenic plants (Fig. 3, panel a). When treated with 1  $\mu$ M IAA, which induces well-separated lateral root primordia, the frequency of primordia initiated per unit length of the main roots was not altered (mean of 1.08 initials/mm with a standard deviation of 0.09 in wild-type compared with 1.14+/- 0.07 and 1.09+/-0.13 in the two transgenic lines examined). However, growth and development of lateral roots following induction by 10  $\mu$ M IAA. was markedly accelerated in the *cdc2aAt::cyc1aAt* transformants, giving rise to a much enlarged root system (Fig. 3, panel b). Enhanced root growth in *cdc2aAt::cyc1aAt* plants following IAA treatment superficially resembles the *alf1* phenotype (Celenza *et al.*, *Genes & Development*, 9:2131, 1995) and these plants have elevated levels of *cyc1aAt* transcripts but in contrast to *cdc2aAt::cyc1aAt* transformants, *alf1* plants initiate supernumerary lateral roots. The several-fold greater gain of fresh weight in IAA-treated *cdc2aAt::cyc1aAt* plants compared to equivalent wild-type controls was accompanied by marked increased in DNA content and dry weight (Table 3). Confocal microscopy confirmed that the enhanced growth response to IAA, which was also observed in several lines showing weaker *cdc2aAt::cyc1aAt* expression, did not reflect transgene stimulation of cell vacuolation or elongation. Thus, ectopic cyclin expression enhances root growth by stimulation of cell division activity in meristems, thereby increasing the rate of cell production without altering meristem organization.

The data above indicate that *cdc2aAt::cyc1aAt* expression is sufficient to enhance growth from established apical meristems, suggesting that cell cycle activity regulates meristem activity. However, the failure to induce gratuitous organ primordia by ectopic expression of *cyc1aAt* under control of the *cdc2aAt* promoter implies additional control points in the generation of a new apical meristem, either through post-translational regulation of cyclin-dependent protein kinase activity or the operation of parallel regulatory pathways. In most animal cells, the commitment to cell division occurs late in G1 (Pardee, A.B., *Science*, 246:603, 1989), and cyclin D1 and cyclin E are rate-limiting for G1 progression in cultured cells (Ohtsubo and Roberts, *Science*, 259:1908, 1993; Quelle *et al.*, *Genes Dev.*, 7:1559, 1993; Resnitzky and Reed, *Mol. Cell Biol.*, 15:3463, 1995). Elevated levels

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of cyclin D1 are observed in several tumors (Motokura *et al.*, *Nature*, 350:512, 1991; Rosenberg *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:9638, 1991; Withers *et al.*, *Mol. Cell Biol.*, 11:4864, 1991) and ectopic expression in transgenic mice promotes hyperplasia and adenocarcinomas (Wang *et al.*, *Nature*, 369:669, 1994).

- 5 In contrast, ectopic expression of *cyclAAt* did not result in neoplasia but stimulated organized growth, without altering meristem organization or size as monitored by confocal microscopy. Moreover, morphology of the transgenic plants was not altered and increased growth was accompanied by accelerated organ development. Thus, cyclin expression is a crucial, limiting upstream factor in an intrinsic regulatory hierarchy
- 10 governing meristem activity, organized growth and indeterminate plant development. This regulatory hierarchy, which is distinctly different from that in animals, where determinate development limits proliferative growth, exemplified by the strict morphogenetic control of cell division during muscle differentiation (Halevy *et al.*, *Science*, 267:1018, 1995; Skapek *et al.*, *Science*, 267:1022, 1995), may underlie the
- 15 striking plasticity of plant growth and development (Drew, M.C., *New Phytol.*, 75:479, 1975). Cyclin abundance may function as a rheostat to allow flexible growth control in response to changes in the environment such as nutrient availability.

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Table 1  
Root apical growth

	Plant line		Rate [ $\mu\text{m}\cdot\text{h}^{-1}$ ]	% of wild-type	n
5	Col-0	(-)	254.1	100	57
	2A5	(-)	253.1	99.6	56
	3A	(+)	341.4*	134.4	20
	3A	(-)	259.4	102.1	30
10	4A3	(+)	291.6*	114.8	47
	6A	(+)	354.1*	139.5	20
	6A	(-)	252.4	99.3	16
	7A	(+)	344.9*	135.7	24
	7A	(-)	249.8	98.3	19
15	8A	(+)	335.4*	131.9	21
	8A	(-)	258.6	101.8	31
	11A1	(-)	258.8	101.8	45
	2A5	(-)	253.1	99.6	56

Table 1 shows a comparison of root apical growth rates. Plant line = independent transformants (except for Col-0). (+) = plants that show enhanced growth phenotype due to presence of adequate levels of cyclin-encoding nucleic acid. (-) = plants that have lost introduced cyclin-encoding nucleic acid or do not exhibit sufficient cyclin expression for enhanced growth. Rate = rate of displacement of root apex per unit time. (\*denotes values significantly different from Wild-Type growth rate.) n = number of individual plants analyzed.



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Table 2

Cell Type	Col-0 (wild type)		7A (transgenic)		8A (transgenic)	
	Size [ $\mu\text{m}$ ]	n	Size [ $\mu\text{m}$ ]	n	Size [ $\mu\text{m}$ ]	n
5 Epidermis	137	37	129	34	158	12
Cortex	159	31	135*	7	160	9
Endodermis	109	23	90*	22	107	11
Pericycle	73	26	67	19	71	57

Table 3

Growth of seedling root system

10

Plant line	Fresh weight [mg]		Dry weight [mg]		DNA per root [ $\mu\text{g}$ ]	
	3d	6d	3d	6d	3d	6d
Col-0	11	25	1.7	2.4	5	14
15 4A3	31	136	4.5	15.4	8	35
6A	30	155	4.2	19.3	10	46
7A	24	156	3.5	16.8	9	38
8A	18	134	2.5	12.8	8	33

Table 2 shows a comparison of cell size; and Table 3 shows a comparison of root growth after IAA treatment in wild-type and in transgenic Arabidopsis lines containing the *cdc2aAt::cyclAAt* gene fusion. The lines 3A, 6A, 7A, 8A are heterozygous T1 populations with more than one introduced transgene; (+) denotes plants with increased *cyclAAt* transcript levels, (-) plants with wild-type *cyclAAt* transcript levels. The following T2 lines are homozygous for *cdc2aAt::cyclAAt*: 2A5, 4A3 and 11A1: constitutive *cyclAAt* expression in 4A3, but not in 2A5 and 11A1, exceeds IAA-induced wild-type levels (Fig. 1). n. number of plants analyzed. \*. means that are significantly different from the wild-type; for a,  $P < 0.001$ , for b,  $P < 0.01$ . Fresh weight = weight of freshly excised root system. Dry weight = weight after 24 hours of drying.

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The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

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What is claimed is:

1. A method of producing a genetically modified plant characterized as having increased growth and yield as compared to the corresponding wild-type plant, said method comprising:
  - 5 contacting plant cells with nucleic acid encoding a cyclin protein, wherein said nucleic acid is operably associated with a regulatory sequence, to obtain transformed plant cells;  
producing plants from said transformed plant cells: and  
selecting a plant exhibiting said increased yield.
- 10 2. The method of claim 1, wherein the genetically modified plant exhibits increased root growth.
3. The method of claim 1, wherein the genetically modified plant exhibits increased shoot growth.
4. The method of claim 1, wherein the cyclin is cycl1aAt.
- 15 5. The method of claim 1, wherein the regulatory sequence is a promoter.
6. The method of claim 5, wherein the promoter is selected from the group consisting of constitutive promoters and inducible promoters.
7. The method of claim 1, wherein the contacting is by physical means.
8. The method of claim 1, wherein the contacting is by chemical means.

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9. The method of claim 1, wherein the plant cell is selected from the group consisting of protoplasts, gamete producing cells, and cells which regenerate into whole plants.
10. The method of claim 1, wherein said nucleic acid is contained in a T-DNA  
5 derived vector.
11. A plant produced by the method of claim 1.
12. Plant tissue derived from a plant produced by the method of claim 1.
13. A seed derived from a plant produced by the method of claim 1.
14. A method of producing a plant characterized as having increased growth and  
10 yield, said method comprising contacting a plant with an agent which elevates cyclin expression above cyclin expression in a plant not contacted with the agent.
15. The method of claim 14, wherein increased growth and yield results from increased root growth.
- 15 16. The method of claim 14 wherein increased growth and yield results from increased shoot growth.
17. The method of claim 14, wherein the cyclin is *cyc1aAt*.
18. The method of claim 14, wherein the agent is a transcription factor.
19. The method of claim 14, wherein the agent is a chemical agent.

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20. A method of providing increased transcription of a nucleic acid sequence in plant tissue, wherein said method comprises:
- 5 growing a plant having integrated in its genome a nucleic acid construct comprising nucleic acid encoding a cyclin protein, wherein said nucleic acid is operably associated with a tissue specific promoter whereby expression of said cyclin-encoding nucleic acid is increased in said plant tissue.

FIG. 1A

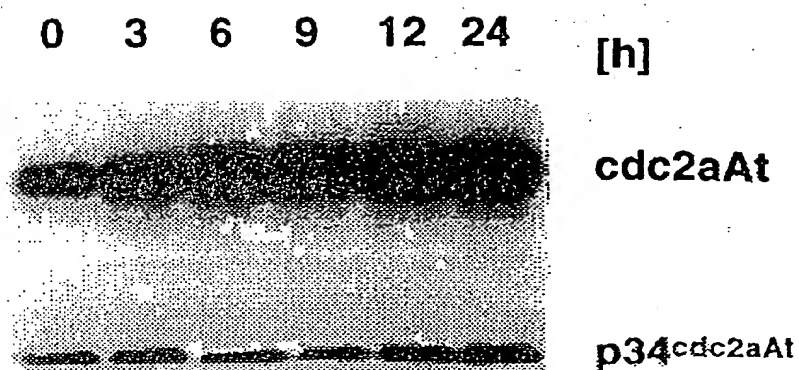
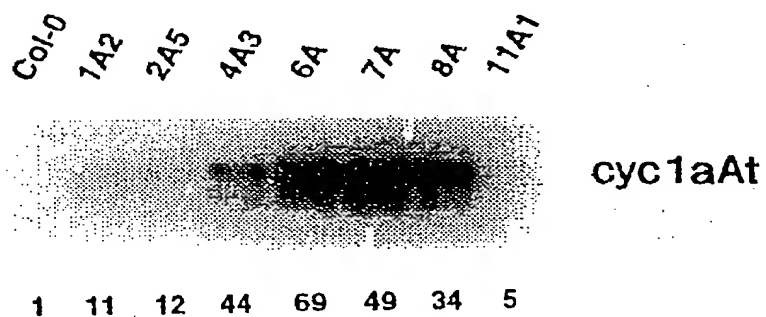


FIG. 1B



FIG. 1C



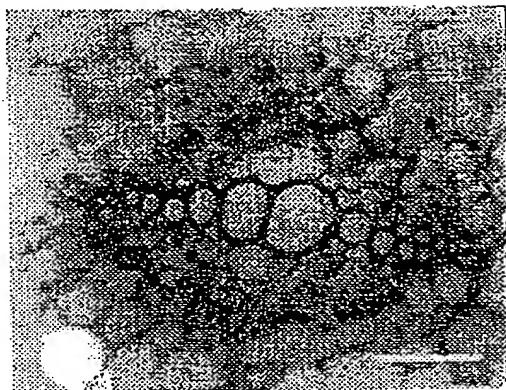


FIG. 2A

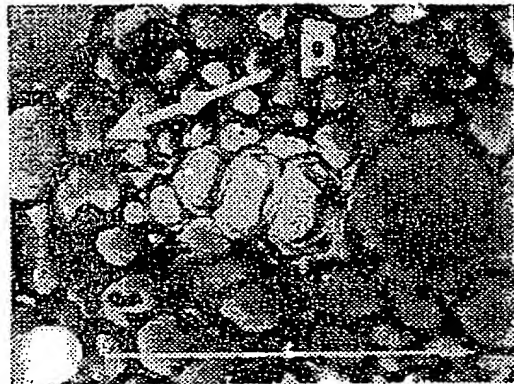


FIG. 2B

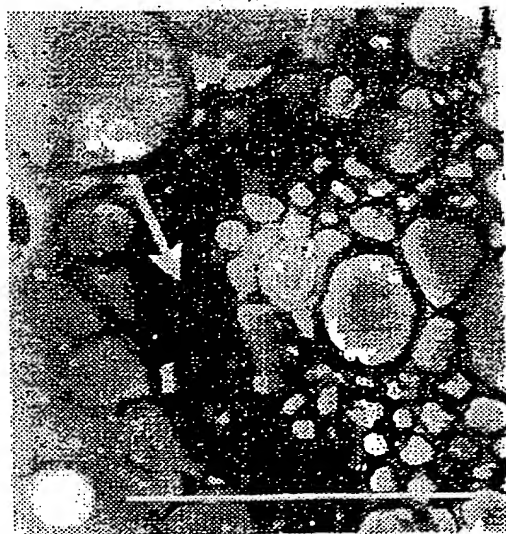


FIG. 2C

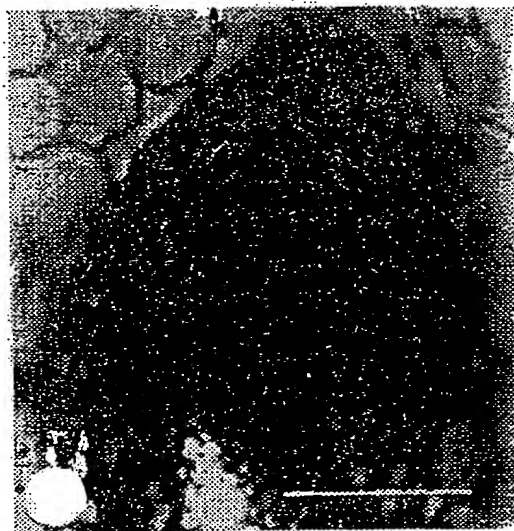


FIG. 2D



FIG. 2E

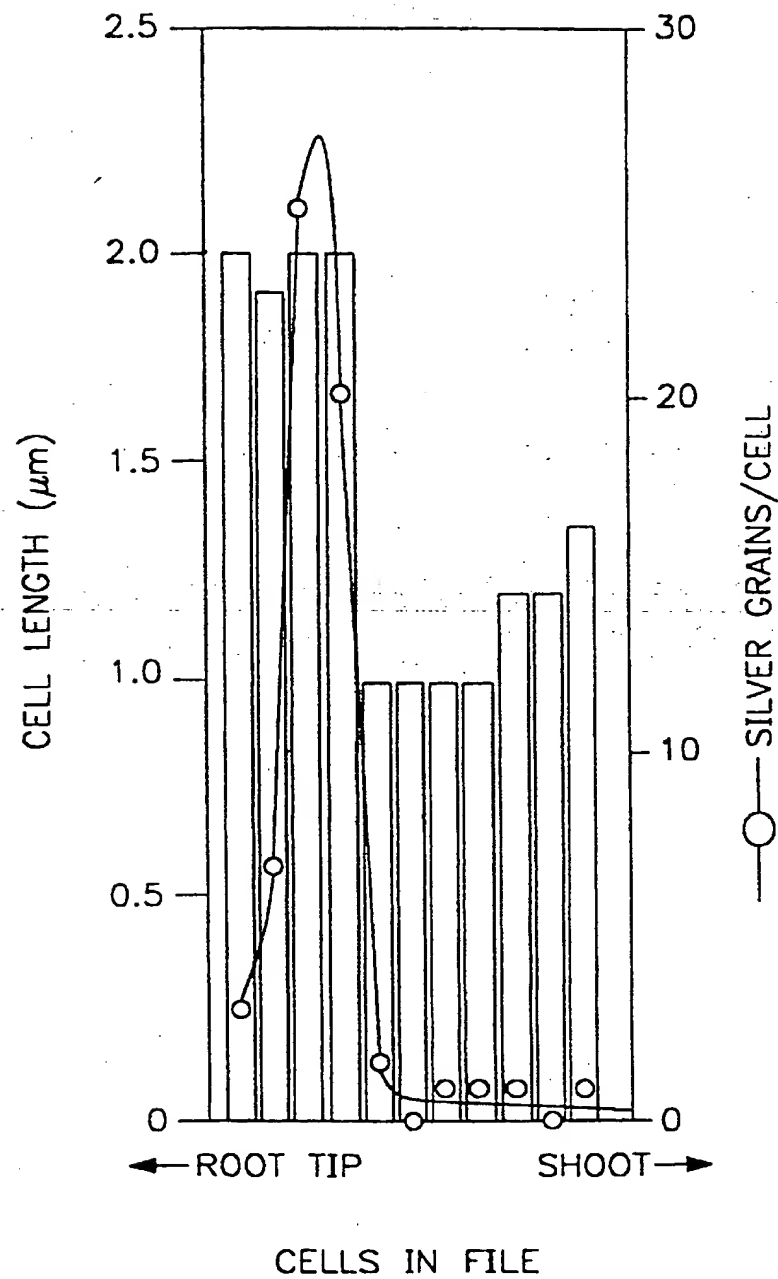


FIG. 2F

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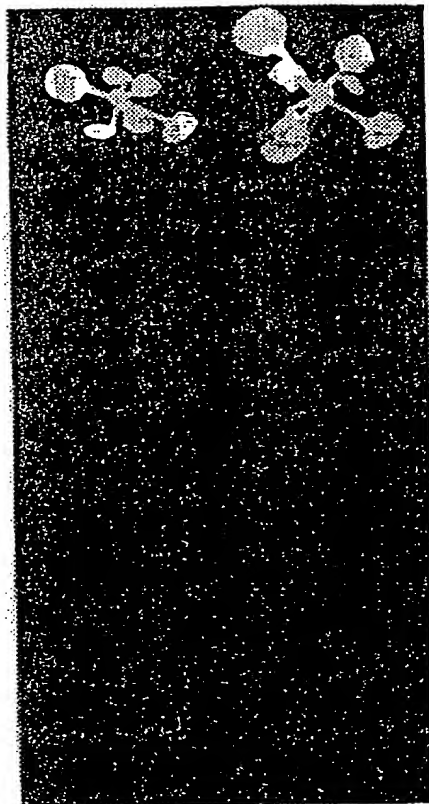


FIG. 3A

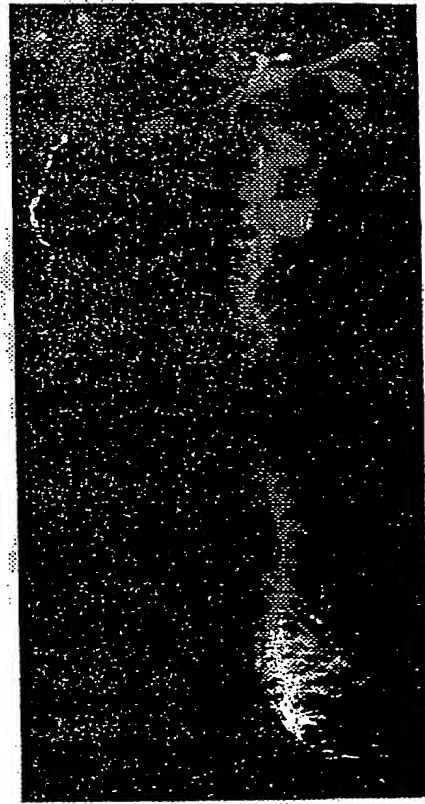


FIG. 3B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12656

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 800/205, 250; 536/23.6, 24.1; 435/172.3, 320.1, 419

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, 250; 536/23.6, 24.1; 435/172.3, 320.1, 419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CABA, CAPLUS, BIOSIS, MEDLINE

search terms: cyclin, cell cycle protein, mitogen, mitosis

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAWAOKA et al. Growth Stimulation of Tobacco Plant Introduced the Horseradish Peroxidase Gene prxCla. Journal of Fermentation and Bioengineering. 1994. Vol. 78, No. 1, pages 49-53	1-20
Y	FERREIRA et al. Control of cell proliferation during plant development. Plant Molecular Biology. 1994. Vol. 26, pages 1289-1303.	1-20
Y	HEMERLY et al. Genes regulating the plant cell cycle: Isolation of a mitotic-like cyclin from Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. April 1992. Vol. 89, pages 3295-3299.	1-20

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 OCTOBER 1997

Date of mailing of the international search report

24 OCT 1997

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12656

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HATA et al. Isolation and characterization of cDNA clones for plant cyclins. The EMBO Journal. 1991. Vol. 10, No. 9, pages 2681-2688.	1-20

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12656

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C12N 5/00, 5/04, 15/00, 15/09, 15/29, 15/63, 15/64, 15/82; A01H 1/00, 3/00, 4/00, 5/00